

CONTINUATION/DIVISIONAL APPLICATION TRANSMITTAL

(Rule 53(b) Continuation or Divisional)

☐ DUPLICATE

Address to

Assistant Commissioner for Patents

Attorney Docket No.:

REF/Sundrehagen/
764

Box PATENT APPLICATION

First Named Inventor:

Sundrehagen et al.

Washington, D.C. 20231

Total Pages:

This requests a ☒ Continuation or ☐ Divisional application under 37 CFR 1.53(b) of prior application:

Appl. No.:

09/201,764

Group Art Unit:

1641

Filed on:

December 1, 1998

Examiner:

J. Hines

Entitled:

ASSAY

- ☐ 1. The entire disclosure of the pending, prior application is hereby incorporated by reference.
- ☒ 2. Submitted herewith is a copy of the complete prior application as filed.
- ☐ 3. This application is filed by fewer than all the inventors named in the prior nonprovisional application, 37 CFR 1.53(b)(1). **DELETE** the following inventor(s): _____.
- ☒ 4. Submitted herewith is a copy of the signed Oath/Declaration from the prior application.
- ☒ 5. Small entity status was established in the prior application, and is still proper and desired.
- ☒ 6. A three month Petition for Extension of Time is filed concurrently in the prior application.
- ☒ 7. The Commissioner is authorized to credit any overpayment and charge any deficiency in any fees required under 37 CFR 1.16 and/or 1.17 to Deposit Account No. 02-0200.
- ☒ 8. A check in the amount of \$1076.00 is submitted herewith.
- ☐ 9. Insert before the first sentence of the specification: -- This application is a ☒ Continuation ☐ Division of nonprovisional application serial number 09/201,764 filed December 1, 1998. --
- ☐ 10. Cancel in this application original claims _____ of the prior application before calculating the filing fee. At least one independent claim is retained.
- ☒ 11. The prior application is assigned of record to: AXIS BIOCHEMICALS ASA.
- ☐ 12. Priority is claimed based on each foreign application so listed in the Oath/Declaration.
- ☐ 13. A Preliminary Amendment is enclosed.
- ☐ 14. Other: _____.

THE FILING FEE IS CALCULATED AS FOLLOWS:

Basic Fee:

\$760.00

Total Claims:

49

- 20 =

29

X \$18 =

522.00

Independent Claims:

1

- 3 =

0

X \$78 =

0.00

Correspondence Address:

BACON & THOMAS, PLLC
625 Slaters Lane, 4th Floor
Alexandria, VA 22314-1176

Multiple Dependent Claim (add \$260.00):

Subtotal:

1,282.00

50% Reduction if Small Entity Status:

641.00

Phone: 703-683-0500

Fax: 703-683-1080

Total:

641.00

Date:

October 13, 1999

Name:

Richard E. Fichter

Signature:

Richard E Fichter

Reg. No.

26,382

VERIFIED STATEMENT (DECLARATION) BY A SMALL BUSINESS CLAIMING SMALL ENTITY STATUS UNDER 37 CFR 1.9(F) AND 1.27(c)

Applicant or Patentee: Erling SUNDREHAGEN et al.

Docket #: REF/Sundrehagen/68828

Serial or Patent Number:

Group Art Unit:

Filed or Issued: December 1, 1998

Examiner:

Title: ASSAY

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

Name of Concern: **AXIS BIOCHEMICALS ASA**

Address: Ulvenveien 87, P.O. Box 206 Økern, N-0510 Oslo, Norway

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the matter described in:

- ☐ The specification filed herewith, with the title as listed above.
- ☒ The patent application identified above.
- ☐ The PCT International patent application identified above.
- ☐ The patent number identified above.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention must file separate verified statements averring to their status as small entities, and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern who would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e). Each person or organization having any rights in the invention is listed below:

- ☐ no such person, concern or organization.
- ☐ each such person, concern or organization listed below:

FULL NAME:	<input type="checkbox"/> Individual <input type="checkbox"/> Small Business Concern <input type="checkbox"/> NonProfit Organization
ADDRESS:	

FULL NAME:	<input type="checkbox"/> Individual <input type="checkbox"/> Small Business Concern <input type="checkbox"/> NonProfit Organization
ADDRESS:	

☐ See attached sheet for additional person(s) concern(s) or organization(s).

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which the verified statement is directed.

Name and Title ERLING SUNDREHAGEN, V.P. R&D.	Date Jan 3, 1999
Address Ulvenveien 87, 0510 OSLO	Signature <i>E. Sundrehagen</i>

68828/001.602

Assay

The present invention relates to assay methods for the determination of cobalamin or vitamin B₁₂ in a body fluid and in particular to assay methods for the metabolically active pool of cobalamin.

Cobalamin or vitamin B₁₂ is a water soluble vitamin which forms part of the vitamin B complex found in foods. The core molecule consists of a corrin ring of four pyrole units which surround the essential cobalt atom. Cobalamin is the only vitamin which cannot be synthesised by animals or plants and must be absorbed from food in the gut. It can however be stored in the liver. It is synthesised by micro-organisms, in particular by anaerobic bacteria and yeasts.

Cobalamin functions *in vivo* as a co-enzyme and cobalamin enzymes catalyse three types of reaction; (i) intra-molecular rearrangements, for example, the formation of succinyl CoA from L-methylmalonyl CoA, (ii) methylations, for example, the formation of methionine by methylation of homocysteine and (iii) reduction of ribonucleotides to deoxyribonucleotides in some micro-organisms. In mammals, only two enzymic reactions, those specifically mentioned in (i) and (ii) above are known to require cobalamin as a co-enzyme.

In the process of digestion, a salivary protein called haptocorrin, hereinafter referred to as HC (which is also referred to in the art as R-binder or transcobalamins I and III collectively) binds cobalamin in the upper gastrointestinal tract forming a complex which passes through the stomach. Pancreatic enzymes digest the cobalamin-haptocorrin (holo-HC) complex in the ileum, liberating cobalamin which is then bound to a protein called intrinsic factor, which is secreted by the gastric mucosa, to form a further complex. The cobalamin-intrinsic factor complex binds to a specific

receptor in the lining of the terminal ileum, whereupon it is dissociated by a releasing factor and the cobalamin transported actively across the membrane of the ileum into the blood stream.

Cobalamin does not circulate in the body in a free form in an appreciable amount. Probably 99% or so of cobalamin is bound by one of the transcobalamin proteins (TC I-III) or albumin.

The protein believed to be responsible for transporting cobalamin to target tissues is transcobalamin II (TC II), a critical trace protein without which cobalamin cannot cross cell membranes. Despite this important metabolic function only about 6-25% of cobalamin in the serum is bound to TC II and most is carried by HC. TC II is a single chain polypeptide of 45 kDa found primarily in serum, seminal fluid and cerebro-spinal fluid. Cobalamin bound TC II or holo-TC II, attaches to specific receptors on cell membranes and once bound, the holo-TC II complex is taken into cells by pinocytosis.

TC II is synthesised by the liver, vascular endothelium, enterocytes, macrophages and fibroblasts and circulates predominantly as apo-TC II, i.e. lacking bound cobalamin. It has a short half life of approximately 90 minutes.

Less than a quarter of the total plasma cobalamin is associated with TC II. The rest is bound to the other transcobalamins or albumin as mentioned above.

Since cobalamin must be absorbed from food, any conditions which result in impaired gastric function, for example, gastroenteritis or conditions resulting in gastric atrophy, or an inability to produce functional haptocorrin, intrinsic factor, releasing factor, TC II or TC II receptors, can result in impaired uptake of cobalamin and resultant deficiency.

Certain population sub-groups, for example the aged, pregnant women, patients with chronic or acute

gastrointestinal disease, those suffering from certain autoimmune diseases, those with a family history of pernicious anaemia and AIDS sufferers, are particularly prone to cobalamin deficiency.

The clinical manifestations of cobalamin deficiency are varied and numerous but primarily involve, anaemia, megaloblastic haematopoiesis and functional and structural disorders of the nervous system. Around 60% of individuals diagnosed as being deficient in cobalamin are anaemic, but in many, neurological symptoms are the only clinical signs observed. Around 10% of patients exhibit psychiatric symptoms and around 40% exhibit both neurological and psychiatric symptoms.

Early diagnosis of cobalamin deficiency is crucial to ensure a good prognosis for patients, since some of the manifestations of cobalamin deficiency, particularly the neuropsychiatric effects, are irreversible if not detected and alleviated by cobalamin therapy quickly.

It is desirable therefore to accurately assess the cobalamin level of an individual in an expedient and efficient manner, with a view to establishing whether or not the individual may be suffering from cobalamin deficiency.

Measurement of total plasma cobalamin ie. cobalamin (and cobalamin like substances) bound to any one of the transcobalamin (TC) proteins I, II and III, has been used in attempts to assess cobalamin deficiency. This technique results in a broad based concentration distribution within a population which is considered to be normal and hence produces a wide reference range. Within individuals however, the range of available cobalamin considered to be normal for that individual, is very narrow. It has been observed that although an individuals metabolically active cobalamin concentration has moved outside their own reference range, their total plasma cobalamin content remains within the range considered to be normal for the population. Under such

circumstances, cobalamin deficiency can go undetected. Such an unreliable method is clearly undesirable and it is well recognised that such serum or plasma cobalamin measurements have low diagnostic sensitivity and specificity.

Microbial assays involving micro-organisms dependent upon cobalamin for growth, have been developed and used in measuring plasma cobalamin concentration, but in addition to the difficulty of estimating the appropriate reference range, these methods require extraction and conversion of the cobalamins which is very time consuming, troublesome and wholly unsuited for rapid laboratory screening.

Alternative methods for assessing cobalamin deficiency involve measuring the accumulation of metabolites in the plasma which require cobalamin for their conversion. Plasma methylmalonate and plasma homocysteine levels increase in cobalamin deficient individuals (Chanarin, The megaloblastic anaemia; London, Blackwell Scientific Publications, 1991) and make good candidate molecules for correlation with vitamin B₁₂ deficiency. Methods based on homocysteine assessment have been shown, however, to be complicated, impractical and show poor specificity and sensitivity. Whilst methods based on methylmalonate measurement are accurate and reliable, they are cumbersome and require analysis by combined gas-chromatography/mass-spectrometry and are hence expensive and again unsuitable for routine clinical screening (Nexo et al. (1994) Scand. J. Clin. Lab. Invest. 54:61-76).

It has also been suggested that measurement of TC II bound cobalamin as opposed to total plasma cobalamin may provide a reliable clinical indicator of the likelihood of cobalamin deficiency (Herbert et al. (1990) Am. J. Hematol. 34:132-139; Wickramasinghe and Fida (1993) J. Clin. Pathol. 46:537-539; US Patent 4680273). However, such efforts to determine holo-TC II

concentration have to date been mostly indirect, estimating holo-TC II concentration as the difference between total plasma cobalamin and the cobalamin concentration of TC II depleted plasma.

Such TC II depletion may be accomplished by adsorption to ammonium sulphate (Carmel (1974) *Am. J. Clin. Pathol.* 62:367-372), microsilica (Herzlich & Hubert (1988) *Lab. Invest.* 58:332-337; Wickramasinghe & Fida (1993) *J. Clin. Pathol.* 46:537-539), microfine glass (Vu et al. (1993) *Am. J. Hematol.* 42:202-211) or immobilized anti-TC II polyclonal antibodies (Lindemans et al. (1983) *Clin. Chim. Acta* 132:53-61). The concentration of cobalamin in total plasma and the depleted fraction is performed by methods well known in the art such as radio or enzyme immunoassay techniques. These methods are unsuitable for routine screening automated or not automated because they are complex and time consuming and because the low degree of specificity of the adsorptive materials used results in insufficient separation of holo-TC II and holo-HC resulting in an overestimation of holo-TC II. Lot-to-lot variation of the adsorptive material introduces further errors and most importantly, the subtraction of one large volume from another large volume results in unacceptable inaccuracies and unreliability.

The other attempts to assess TC II have involved separating TC II from other serum components, including the TC I and TC III, using its lipophilicity. Thus Kapel et al. (1988) *Clin. Chim. Acta* 172:297-310, Benhayoun et al. (1993) *Acta Haematol.* 89:195-199 and Toft et al. (1994) *Scand. J. Clin. Lab. Invest.* 54:62 disclose methods for separating TC II from other transcobalamins using heparin sepharose, silica gel or cellulose respectively. These methods however suffer from the same disadvantages as the indirect methods since they rely on the same adsorptive materials. Also, the low plasma concentration of holo-TC II renders these

methods unsuitable for combination with existing methods of cobalamin quantification. The normal range of holo-TC II is 35-160 pM and values below 35 pM would generally be considered as indicative of cobalamin deficiency. The reported analytical sensitivity of most routine methods for plasma cobalamin is about 40 pM but in practice it is often much higher, typically around 90 pM. Hence, normal plasma levels of holo-TC II are below or near the sensitivity limit of the routine methods for cobalamin quantification.

Possibly the most accurate method currently recognised for determining TC II bound cobalamin involves adsorbing TC II to silica and then assaying the bound fraction for cobalamin content using either an immunoassay as described for example by Kuemmerle *et al.* (1992) Clin. Chem. 38/10: 2073-2077, or a microbiological assay, the latter apparently producing the best results. This method requires an entire working day to perform only twenty assays. It is very expensive and impractical and poorly suited to routine clinical diagnostic laboratory investigations.

Thus, there exists a great need for improved methods of assessing the level of metabolically active cobalamin in a body fluid, with a view to correlating the cobalamin level with the likelihood of cobalamin deficiency, which are amenable to routine clinical diagnostic application.

Thus, according to a first aspect, the present invention provides an assay method for the determination of transcobalamin II bound cobalamin in a body sample, comprising contacting a cell free sample of a body fluid with an immobilised or immobilizable specific binding ligand for TC II or holo TC II, separating a ligand bound fraction from a non-ligand bound fraction and measuring the holo-TC II or TC-II bound cobalamin content therein.

By a specific binding ligand is meant one which

binds to TC II (ie. apo-TC II and holo-TC II) or holo TC II by virtue of its specific chemical structure or conformation and not simply by virtue of an overall physico-chemical property (such as lipophilicity) which may be common to many components of a body fluid sample. The binding affinity and specificity of the ligands suitable for use in the method preferably allow for a 3-fold, more preferably a 5-fold and even more preferably a 10-fold and most preferably a greater than 10-fold concentration of the TC II or holo-TC II in the sample and hence such a method is able to give accurate and reliable values for holo-TC II in the lower normal range of holo-TC II and also in the sub-normal range. Such a separation and concentration of the target molecules is not possible with currently existing methods which are unable to efficiently separate TC II or holo-TC II from HC or holo-HC. The ability to concentrate the TC II or holo-TC II at least 3-fold allows the use of automated analytical equipment in the performance assay. Without such a concentrating step, the amount of analyte present in a sample is likely to be below the lower detection limit. Such automated analytical equipment typically requires between 40 and 100 μ l of sample in a total volume of typically 150 μ l or greater for its operation. Thus, a low concentration analyte is diluted even further for analysis. By using the assay method of the invention however, the analyte is concentrated at least 3-fold. Since using such automated analytical equipment typically involves a control range of 100-700 pM with a lower sensitivity limit of about 40 pM but a practical lower limit of around 90 pM, a 5-fold concentration facilitates the measurement of holo-TC II at down to 18 pM and 10-fold concentration facilitates measurement down to 9 pM holo-TC II. Since the present invention facilitates concentration of greater than 10-fold it will be easily understood by the skilled person the extent to which sensitivity is enhanced making it a very

powerful technique indeed.

The ability to concentrate the analyte so that it is amenable to analysis by such automated procedures is an important advantage of the present assay method.

Preferably from a 600 μ l starting sample of body fluid, a volume of up to 150 μ l, more preferably up to 100 μ l and most preferably less than 60 μ l will be generated which may then be analysed, optionally using automated procedures.

Any TC II specific binding ligand may be used in the method of the invention as either a capturing, concentrating and separating ligand or a detecting ligand, and depending on the specific embodiment of the invention may bind to TC II in both the apo and holo form or it may bind specifically or preferentially to the holo-form. The TC II binding ligand will preferably exhibit a high degree of selectivity and specificity towards TC II and will exhibit low or more preferably, essentially no affinity towards other TC proteins, ie. TC I or III, in either apo or holo form, or any other cobalamin-binding protein. If the TC II binding ligand is a holo-TC II specific binding ligand, the same properties are exhibited with the additional requirement that low or preferably no affinity is shown towards apo-TC II.

The TC II or holo-TC II binding ligand will generally be either an antibody, an antibody fragment or a compound with an affinity for TC II or holo-TC II respectively such as a polypeptide, an oligopeptide, a small organic chemical, etc. Other binding ligands may be a specific binder selected from a combinatorial chemistry or phage display library or a specifically binding sequence of DNA or RNA.

If the binding ligand is an antibody it may be polyclonal but will preferably be monoclonal. Monoclonal antibodies can be generated with much greater specificity and uniformity than polyclonal antibodies

and this reduces cross-reactivity with other components of the body fluid, in particular other transcobalamins and where appropriate, the alternative conformation ie. the apo form of the target analyte. The uniformity and reproducibility offered by monoclonal antibodies relative to polyclonal antibodies ensures a greater accuracy which is vital for an assay wherein the analyte is in such low concentration. Alternatively, it may be an antibody fragment for example F(ab), F(ab')₂ or F(v) fragment. The antibodies or antibody fragments may be monovalent or divalent and may be produced by hybridoma technology or be of synthetic origin, and generated by recombinant DNA technology or chemical synthesis. Single chain antibodies or other antibody derivatives or mimics could for example be used. The antibody may be directed or raised against any epitope, component or structure of the TC II or holo-TC II protein as appropriate.

Suitable antibodies for use as binding ligands in the present invention are disclosed for example by Quadros et al. (1996) Biochem. Biophys. Res. Commun. **222**:149-154; McLean et al. (1997) Blood **89**(1):235-242.

Where the ligand is immobilized, this may for example be on the surface of a solid, e.g. a filter film or sheet or the lumen of a tube, however immobilization on particles, e.g. microspheres such as those produced by Dyno Industrier ASA, Norway, is especially preferred. Such particles may be porous or non-porous and if desired may be provided with means which enable them to be collected, e.g. by inclusion with the particles of magnetically responsive material, for example superparamagnetic iron oxide crystals. Such magnetically responsive microbeads are available from Dyno Industrier ASA as well as from Dynal AS, Norway, Bang Particles, USA and Prolabo, France. Where the ligand is to be immobilizable as opposed to immobilized, this may be achieved by coupling the ligand to a member

of a specific binding pair (e.g. biotin/streptavidin) and using the other member of the binding pair, optionally bound to a substrate, to agglomerate or otherwise immobilize the ligand or the ligand:TC II or ligand: holo-TC II complexes, prior to separation of the ligand bound fraction from the non bound fraction in the performance of the method of the invention.

It is well known in the art to immobilise affinity molecules e.g. antibodies and antibody fragments for separation purposes, for example by binding or coupling the ligands, optionally by means of a linker, to any of the well known solid supports or matrices which are currently widely used or proposed for separation or immobilisation on columns and any known method in the art could be used. Such solid phases may take the form of particles, sheets, gels, filters, membranes, fibres or capillaries or microtitre strips, tubes or plates of wells etc and conveniently may be made of glass, silica, latex or a polymeric material. Techniques for binding the ligand to the solid support are also extremely well known and widely described in the literature.

Coupling of the ligand to a substrate or to one member of a specific binding pair may be achieved using conventional techniques.

The method of the invention, if the assay is performed as a competitive binding assay, will generally involve addition to the sample of a labelled compound which competes for binding to the ligand. Generally, it will be preferred to use labelled holo-TC II in this regard. The label used may be any label which can be determined directly or indirectly, e.g. a chromophore (which term is used to include fluorophores), a radiolabel (generally covalently bound or chelate-bound), an enzyme or enzyme substrate, a magnetic label, etc.

A preferred embodiment, the method of the present invention involves contacting an immobilised or an

immobilisable TC II or holo-TC II binding ligand with the sample under investigation;

separating a ligand-bound fraction from a non-ligand-bound fraction;

dissociating bound cobalamin from the holo-TC II molecules in the bound fraction and determining the concentration of cobalamin released, preferably with dissociation being so affected that the concentration of the released cobalamin is at least 3 times, preferably 5 times and even more preferably 10 times greater than the concentrations of holo-TC II in the initial sample.

The essence of this aspect of the invention is the separation and concentration of TC II or holo-TC II which allows standard methods of cobalamin determination to be usefully employed in the method. As indicated above, in the absence of such a concentration step state of the art methods are not suitable for cobalamin determination since it exists at such a low concentration. Any appropriate means for releasing cobalamin from the holo-TC II may be used but conveniently heat or changing the pH of the surrounding medium may be used in this regard. The different cobalimin forms may be converted to the less light sensitive cyanocobalamin by treatment with KCN. Any appropriate means for free cobalamin determination may be employed in the method of the invention for example a competition assay performed by contacting an immobilised binding partner for cobalamin with the dissociated cobalamin of the sample in the presence of labelled ligand which competes with the isolated cobalamin for binding to the immobilised binding partners. A method as described by Kuemmerk *et al.* (1992) Clin. Chem 38:2073-2077 for example would be appropriate. An alternative means for determining free cobalamin is described in US Patent No. 5451508 and involves a immunoassay technique.

In this embodiment of the invention it is preferred

that the binding ligands for TC II are immobilised and bind to both holo and apo-TC II.

In an alternative preferred embodiment, the assay method of the present invention comprises contacting a solid support having immobilised thereon a TC II or holo-TC II binding ligand, with the sample under investigation and also with a non-immobilised ligand,

wherein said immobilised ligand is capable of binding to TC II or holo-TC II, to said non-immobilised ligand or to complexes of said TC II or holo-TC II and said non-immobilised ligand, and said non-immobilised ligand is capable of binding to at least one of said immobilised ligand, TC II or holo-TC II and complexes of said immobilised ligand and TC II or holo-TC II;

wherein if said assay method is a sandwich assay, at least one of said ligands is specific for holo-TC II and if said assay is a competition assay said immobilised ligand is specific for holo-TC II and competitors thereof;

whereby the proportion of said immobilised ligand bound by TC II or holo-TC II, by said non-immobilised ligand or by complexes of said non-immobilised ligand and TC II or holo-TC II is dependent on the amount of holo-TC II present in said sample, and,

said non-immobilised ligand is capable of generating a directly or indirectly detectable signal when bound or when unbound;

separating a bound fraction from a non-bound fraction; and

directly or indirectly determining the non-immobilised ligand bound to the immobilised ligand (the bound fraction) or non-bound and in solution (the non-bound fraction);

where the contacting of the sample and said non-immobilised ligand with the solid support may be performed separately, simultaneously or sequentially, and if performed separately or sequentially, they can be

contacted in either order.

In essence therefore, the alternative preferred embodiment of the method of the invention involves determining the non-immobilised ligand which has either directly or indirectly bound or alternatively failed to bind directly or indirectly to the immobilised ligand. Where the non-immobilised ligand competes for binding to the immobilised ligand with the holo-TC II, a high level of unbound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of unbound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample. Where the non-immobilised ligand binds to the TC II or holo-TC II which is bound in turn to the immobilised ligand, then a high level of bound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of bound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample.

In the method of the present invention therefore, the metabolically active pool of cobalamin is determined by measuring the holo-TC II complex or measuring the amount of cobalamin bound to TC II molecules, present in a body sample which contains a mixture of both apo and holo-TC II and apo and holo-HC (haptocorrin or TC I and III).

A preliminary separation step may be carried out using immobilised cobalamin or an analogue or fragment thereof which selectively binds to the apo-forms of both TC II and haptocorrin (HC). In such a preliminary step, the apo forms of the TC II and HC proteins are bound by the immobilised cobalamin, analogue or fragment thereof and separated from the holo-TC II and holo-HC complexes.

In this case, analysis of the separated holo-forms of TC II then takes place according to any embodiment of the invention as described above but clearly it is most useful where determination of the holo-TC II complex

takes place by other than dissociation of the complex and subsequent determination of the released cobalamin. It will be apparent to the skilled person that if such a preliminary step precedes the above-mentioned alternative preferred embodiment, the requirement for at least one ligand in a sandwich assay to be specific for holo-TC II as opposed to TC II and the requirement for the immobilised ligand in a competition assay to be specific for holo-TC II and competitors thereof as opposed to TC II is no longer relevant since the apo-form of TC II has been captured and is no longer available. Thus, the immobilised or non-immobilised ligands may be specific for holo-TC II or TC II or competitors thereof.

The immobilised cobalamin should not exhibit any significant tendency to become dissociated from its support since this could result in binding to apo-TC II and transformation thereof to holo-TC II. Biotinylated cobalamin binds in a very stable manner to a solid surface with avadin/streptavidin bound thereto, and tethering cobalamin to a support in this manner is convenient for the performance of this step. In fact, when biotinylated cobalamin is used, can be added to the sample under analysis in a non-immobilised form where it binds to the apo-forms of TC II and HC. The sample may then be contacted with a solid surface having a binding partner for the biotin example, avidin, immobilised thereon. A complex of avadin-biotinylated cobalamin-TC II then forms which can be easily isolated from the sample.

In one embodiment of the invention wherein this preliminary separation step has taken place, the holo-TC II pool is subsequently determined by contacting the sample with an immobilised TC II ligand which captures the holo-TC II complex leaving the haptocorrin in solution and then contacting the immobilised holo-TC II with a second TC II ligand which is labelled and thus

detectable. Examples of suitable TC II binding ligands would be non-overlapping monoclonal antibodies or indeed a polyclonal antibody specific for TC II would be suitable in this embodiment for both capture and detection since different epitopes on the TC II molecules are recognised.

In another embodiment of the invention wherein this preliminary separation step has taken place, the holo-TC II molecules compete with the labelled non-immobilised ligand for binding to the immobilised ligand therefor and the amount of holo-TC II is calculated in relation to the amount of labelled non-immobilised ligand bound or not bound to the immobilised ligand.

In a preferred embodiment, in the preliminary separation step, the binding of apo TC II and apo HC to cobalamin, analogues or fragments thereof takes place at a site or in such a manner which inhibits subsequent recognition and binding of the immobilised cobalamin bound TC II by the non-immobilised ligand or binding partner for TC II. In this embodiment there is no need to isolate the holo-TC II and holo-HC from the bound apo forms before performing the assay of the invention. In this embodiment, the site against which the non-immobilised binding partner or ligand is directed is very important and should be an epitope on TC II which becomes masked or shielded or otherwise unavailable for binding when the apo TC II and apo-HC becomes immobilised on the cobalamin, analogue or fragment thereof.

Whether the initial separation step involves using a binding ligand for TC II or it is preceded by a preliminary step using immobilised cobalamin, analogues or fragments thereof, total separation, ie. separation of all the apo and holo TC II proteins, from the sample, is not a requirement of the method and it suffices that a fraction is separated from the sample which comprises at least a portion of the desired TC II subset of

proteins'.

Thus, in certain embodiments, the binding partners or ligands and binding conditions may be selected so as to achieve separation of substantially all of the selected "subset". In this case the non-bound fraction may be regarded as being substantially free of TC II or holo-TC II proteins, i.e. being at least 80%, 90% or 95% free of either TC II or holo-TC II depending upon which component is selected as the basis of fractionation.

In alternative embodiments, the binding partner and conditions may be selected so that only a part of the TC II proteins in the sample are separated into a fraction. In this case, account should be taken of the partial separation in constructing a standard calibration curve for the assay. In this regard, the generation of a standard calibration curve against which the concentration of the holo-TC II detected can be determined uses standard techniques well known in the art.

Thus, if in a fractionation step, a TC II binding ligand is used, at least a portion of the TC II bound cobalamin will be located in the bound fraction and any cobalamin in the sample bound to molecules other than TC II, for example, HC or albumin will be in the non-bound (ie. non-separated fraction or fractions). TC II in the bound fraction may be in either apo and holo form and may be complexed by cobalamin like substances or analogues in addition to cobalamin.

If desired, the method of the invention may involve a further preliminary separation step in which the sample is contacted with an immobilized or immobilizable specific binding ligand for haptocorrin (in apo and holo forms or in holo form alone). In this way any contribution to errors in the TC II-bound cobalamin determination deriving from holo-HC may be reduced and specific binding ligands for TC II or holo-TC II may be used which have some binding affinity for haptocorrins.

Otherwise, as indicated above, since the serum concentration of TC II both in apo and holo form is very low, ligands or binding partners of particularly high specificity and affinity are necessary for the operation of the invention. Also, the affinity constants required of the ligands of the present invention depend upon whether the ligand is specific for TC II or holo-TC II and also its utilization as either a capture or detection ligand, since the serum concentration of TC II is about 0.5-1 nM but the concentration of holo-TC II only 35-160 pM. Thus, for a TC II capturing ligand an affinity constant of at least $10^9 M^{-1}$, preferably greater than $2 \times 10^9 M^{-1}$ and more preferably $10^{10} M^{-1}$ is desirable. For a holo-TC II capturing ligand, an affinity constant of at least $10^{10} M^{-1}$ is desirable, preferably $2 \times 10^{10} M^{-1}$, more preferably greater than $2 \times 10^{10} M^{-1}$ and most preferably greater than $10^{11} M^{-1}$. Clearly the affinity constant required of a detecting binder may be less than that of a capturing ligand due to the concentration effect of the assay.

The degree of cross reactivity of a holo-TC II or TC II binding ligand with HC should preferably be less than 1%, more preferably between 0.1% and 1% and most preferably less than 0.1%. Similarly, a holo-TC II binding ligand should preferably not exhibit a degree of cross reactivity with apo-TC II in excess of 1%, more preferably between 0.1% and 1% and most preferably the cross-reactivity should be less than 0.1%.

By using such high affinity ligands their function is extended beyond that of simply being capture and/or detection ligands and they play a vital role in being able to separate and concentrate TC II protein from the sample for analysis. The binding ligands for TC II or holo-TC II should preferably concentrate the ligand by at least 3-fold, more preferably 5-fold, and even more preferably by at least 10-fold.

In a further embodiment of the assay technique more

akin to a displacement assay than a competition assay, the sample comprising the holo-TC II complex is contacted with a solid phase to which a labelled ligand recognising the same binding sites on the immobilised ligands as holo-TC II, is bound. The holo-TC II in the sample competes with the bound labelled ligand for the sites such that after equilibration of the system, there is a directly proportional relationship between the amount of labelled ligand displaced from the solid support and detectable in solution and the amount of holo-TC II present in the original sample. The labelled ligand may be detected directly or indirectly and may be determined as the amount of labelled ligand bound or not bound to the solid support as appropriate. In effect, in this embodiment, the non-immobilised ligand referred to earlier is bound to the immobilised ligand before application of the sample but such binding should not be construed to mean that the ligand is in any way immobilised.

A further preferred embodiment involves contacting a holo-TC II containing sample with a solid support having holo-TC II immobilised thereon and a labelled un-immobilised holo-TC II specific binder. The free holo-TC II in the sample and the immobilised holo-TC II compete for binding with the labelled non-immobilised ligand and determination of the labelled ligand bound to the solid phase or remaining in solution allows calculation of the holo-TC II concentration. In a particularly preferred embodiment of the present invention the labelled non-immobilised holo-TC II binding ligand is an antibody.

In yet another embodiment, the sample comprising the analyte of interest is contacted with labelled holo-TC II and immobilised ligand. The labelled and non-labelled holo-TC II compete for binding to the immobilised ligand and after equilibrium is reached, the amount of labelled holo-TC II bound to the immobilised

ligand is indirectly proportional to the amount of holo-TC II in the sample of interest. Again, the labelled holo-TC II may be detected directly or indirectly and may be determined as the amount of labelled holo-TC II bound or not bound to the solid support as appropriate.

In a further embodiment of the present invention, a non-immobilised but immobilizable ligand specific for the holo-TC II complex, (e.g. a ligand conjugated to biotin or another member of a specific binding pair) is contacted with the sample to form a holo-TC II/non-immobilised ligand complex. The ligand/holo-TC II complex may then be precipitated from solution using known means and isolated for analysis.

In yet another embodiment of the invention, two labelled binding partners are added to the sample, optionally after a preliminary step such as depleting the sample of the apo forms of TC II and HC. The binding partners in this case are labelled holo-TC II or an analogue or fragment thereof and a labelled binding partner therefor, for example a labelled antibody. In this embodiment a detectable signal is only generated from the labels when they are in close proximity to each other ie. when the two labelled binding partners become bound to each other. Thus, on addition to the sample, the labelled binding partner for holo-TC II may bind either to unlabelled holo-TC II present in the sample in which case no detectable signal is generated or to labelled holo-TC II or an analogue, fragment or variant thereof which binds the labelled TC II binding partner, in which case a detectable signal is generated. Labelling agents such as those of Amersham scintillation proximity assays as described in US Patent No. 4568649 are suitable for incorporation into such an embodiment and the high sensitivity of this procedure is well suited to an assay wherein the analyte exists at such a low concentration. Thus for example, one member of the binding pair could be labelled with a β -emitting nuclide

such as I^{125} or H^3 and the other member is labelled with a suitable scintillant molecule. The emitted β particle loses its energy in its aqueous surroundings unless the scintillant is within $1.5 \mu m$ and thus requires both labelled partners to be bound to each other for a signal to be detectable. The probability of two labelled partners binding each other is determined by the concentration of holo-TC II present in the sample such that the greater the signal generated, the lower the concentration of holo-TC II in the sample.

As used herein, the terms "determining" or "assessing" include both quantitation in the sense of obtaining an absolute value for the amount or concentration of holo-TC II or TC II bound cobalamin in the sample, and also semi-quantitative and qualitative assessments or determinations. An index, ratio, percentage or similar indication of the level or amount of TC II bound cobalamin, for example relative to total cobalamin, may be obtained.

The body sample used in the assay method of the invention may be any cobalamin containing sample e.g. a body fluid or tissue sample, or a suspension etc. Preferably, however the sample will be a body fluid for example, seminal fluid, cerebro-spinal fluid or amniotic fluid, but will generally be a blood derived sample. When this is the case, as the sample used for analysis is preferably cell-free, either serum or plasma may be used. The sample may be treated prior to being used in the assay method of the invention, for example it may be diluted by adding a buffer or other aqueous medium and may be stored or preserved for example by chilling or freezing prior to analysis.

In the practise of the invention, where a bound fraction is separated from an unbound fraction this may be performed by any suitable means, for example, precipitation, centrifugation, filtration, chromatographic methods etc.

For the avoidance of doubt, the term "cobalamin" is used herein synonymously with "vitamin B₁₂" and includes all forms of vitamin B₁₂ (cyanocobalamin; 5-6-dimethylbenzimidazolyl cyanocobamide; methylcobalamine; 5'-deoxyadenosylcobalamin) as may occur and be metabolically active (when appropriately presented) in the body.

As indicated above, in the method of the invention, the determination of the TC-bound cobalamin may be performed by detecting a ligand bound to the separated holo-TC II, or by performing a competition assay using a labelled competitor to holo-TC II whereby the holo-TC II competes with the labelled competitor for binding to a binding ligand therefor, or by detecting cobalamin released from separated holo-TC II.

The detectable ligand may conveniently be labelled by any conventional means, for example with a signal forming label which may be determined by e.g. luminescence, chemiluminescence, colorimetric assessment, fluorescence, radioactivity or by enzymic activity. In effect, any signal forming label known in the art may be used in the method of the invention.

By means of example only, some suitable examples of coloured or fluorescent compounds which may be used to label a binding ligand detectably in the present invention are anthraquinones, azodyes, azine dyes such as oxazines and thiazines, triazines, naturally occurring pigments such as porphyrins, phycobiliproteins, including phycoerythins and phycoquinans, chlorophylls and their analogues and derivatives, carotenoids, acridines, xanthenes including fluoresceins and rhodamines, indigo-dyes, thioxanthenes, coumarins, polymethines including di and tri arylmethines and derivatives thereof of phthalocyanines and metal phthalocyanines.

Similarly, a wide range of radioactive compounds may be used as the signal forming label part of the

reagent used in this invention, among them Iodine-125-labelled compounds.

Alternatively, the binding ligands may be conjugated to natural or synthetic compounds which can produce a chemiluminescent signal which may be assayed in known manner (Cormier, M.J. et al.; Chemiluminescence and Bioluminescence, Plenum Press, New York 1973). Suitable chemiluminescent compounds include luciferin, oxalic esters, 1,2-dioxethane, luminol or derivatives thereof, but are not limited to these. If appropriate, hydrogen peroxide, enzymes e.g. luciferase, or other chemicals may be used to produce the chemiluminescent signal from the signal-producing molecules used.

Strongly-anionic signal forming molecules may not be preferred for use in the method of the invention, since they have a tendency to bind to serum proteins such as human serum albumin (HSA) which may be present in the sample. Particularly suitable examples which may be used are fluorescein isothiocyanate, Rhodamine B or N-(resorufin-4-carbonyl)piperidine-4-carboxylic acid-N-hydroxysuccinimide-ester) or resos.

In accordance with one embodiment of the method of the invention, a fraction comprising at least a portion of the TC II may be separated from a body fluid sample by reacting the body fluid with a binding ligand specific for TC II and then separating the TC II bound fraction from the rest of the sample, thereby isolating and concentrating the target analyte therein. In one embodiment of the invention, a detectable binding ligand directed against holo-TC II may be used to determine the TC II-cobalamin complex present in the separated bound fraction. The holo-TC II binding ligand may be contacted with the sample under investigation prior to, simultaneously with or after contact with the TC II binding ligand and before or after separation of the TC II bound fraction from the rest of the sample.

Correspondingly, a cobalamin containing fraction

(e.g. comprising holo-TC II and holo-HC) may be separated from the rest of the sample by reacting the body fluid under investigation with an immobilised or tethered cobalamin or an analogue or fragment thereof which binds apo-TC II and apo-HC and separating from the bound fraction the rest of the sample which comprises holo-TC II and holo-HC. A detectable TC II binding ligand can then be used to detect the TC II-cobalamin complex present in the separated fraction. The detectable TC II binding ligand may be contacted with the sample under study prior to, simultaneously with or after contact with the bound cobalamin and before or after separation of the bound fraction from the rest of the sample.

In some embodiments, immobilised ligands for one or the other components of the apo and/or holo TC II/HC complexes may be arranged in a column. The body fluid containing the TC II cobalamin complex may be applied to the column and contacted with the binding ligand(s).

The column may be flushed through or washed and an eluent used to allow, if necessary, release of a bound fraction from the column and facilitate its collection. If the contents of the unbound fraction are or are also to be analysed with respect to other constituents, the column should be washed with a buffer or medium administered using a calibrated micropipette to ensure a precise known volume is administered and collected. The volume administered is preferably within 3% of the desired (ie. calibration) volume, more preferably within 1 or 2%. Likewise, if the fraction to be analysed is to be released from the column prior to detection, the eluant used to release the complex should be administered using a calibrated micropipette.

In an alternative embodiment, the binding ligands used to separate a fraction of a sample may be immobilised on a particulate solid phase support, for example latex or polymer beads. To aid manipulation and

separation, magnetic beads may be used and indeed this is a preferred embodiment of the present invention. the term "magnetic" as used herein means that the support is capable of having a magnetic moment imparted to it when placed in a magnetic field. In other words, a support comprising magnetic particles may readily be removed by magnetic aggregation, which provides a quick, simple and efficient way of separating the fractions following ligand binding.

Thus, using the method of the invention, the magnetic particles with the TC II-cobalamin complex attached may be removed onto a suitable surface by application of a magnetic field, for example, using a permanent magnet. It is usually sufficient to apply a magnet to the side of the vessel containing the sample mixture to cause the particles to congregate at the wall of the vessel and so isolate them for further analysis.

Especially preferred are superparamagnetic particles for example those described by Sintef in EP-A-106873, as magnetic aggregation and clumping of the particles during the reaction can be avoided. The well-known magnetic beads made by Bang Particles (US) may be particularly suited to use in the present invention.

In general, besides the sample under evaluation, calibration samples with known holo-TC II complex content will also be assessed in the performance of the assay method of the invention. Such determinations can be used to plot a calibration curve from which the TC II bound cobalamin content of the sample under investigation may be determined. The nature of the calibration samples and selection of conversion or adjustment factors used in the determination of the holo-TC II complex may vary depending, for example, on the particular ligands used in the binding and separation steps of the assay and other aspects of the method which affect the binding and separation of the sample, for example, buffer composition, assay

conditions etc. Typically, calibration samples having holo-TC II contents of 0 to 300 pmol/l will be used. The reference range within which the value for TC II bound cobalamin will generally be found is 30 to 160 pmol/l.

Viewed from a further aspect, the invention provides a kit for a diagnostic assay according to the invention, said kit comprising:

an immobilized or immobilizable specific binding ligand for TC II or holo-TC II;

preferably a holo-TC II solution of known concentration and more preferably a set of such solutions having a range of holo-TC II complex concentrations;

optionally, a release agent to release cobalamin from holo-TC II; and

optionally a labelled ligand.

The assay method of the invention determines the metabolically active pool of cobalamin by determining the holo-TC II complex or isolating the holo-TC II complex and then determining the cobalamin associated therewith and thus provides a convenient method for the determination of cobalamin deficiency before or after the onset of clinical manifestations of cobalamin deficiency. The assay method may be advantageously used prior to the onset of symptoms since it has an accurate predictive value of negative cobalamin balance.

The present invention will now be illustrated by the following non-limiting Examples:

EXAMPLE 1

Monoclonal antibodies specific for TC II are immobilized on magnetizable particles. An aliquot of serum (100-500 μ l) mixed with an equal volume of PBS is allowed to react for 15 min with an excess of the immobilized antibody and an excess of a non-overlapping anti-holo-TC II monoclonal antibody radiolabelled with

I^{125} . The magnetizable particles are sedimented by using a strong magnet, the supernatant is removed, and the particles are washed with PBS.

The radioactivity is measured and the holo-TC II concentration of the sample is determined by interpolation on a standard curve.

EXAMPLE 2

Monoclonal antibodies specific for TC II are immobilized on magnetizable particles. An aliquot of serum (600 μ l) mixed with an equal volume of PBS is allowed to react with the antibody for 30 min. The magnetizable particles are sedimented by using a strong magnet and the supernatant removed. The particles are washed once with PBS and are subsequently treated with sodium hydroxide (0.3 M) containing potassium cyanide (100 μ M), dithiothreitol (15 mM), and a fixed amount of cyanocobalamin radiolabelled with I^{125} in a total volume of 100 μ l for 15 min, to release cobalamin bound to the immobilized holo-TC II and at the same time convert all forms of cobalamin to cyanocobalamin and mix with a fixed amount of labelled cyanocobalamin. A limited amount of Intrinsic Factor in 100 μ l of borate buffer is added and allowed to react for 10 min. The magnetizable particles are sedimented by a strong magnet and 150 μ L of the supernatant removed for measuring radioactivity. The concentration of TC II-bound cobalamin in the serum sample is determined from a standard curve.

Alternatively the released cobalamin may be measured using Abbot's IMx method or similar methods.

EXAMPLE 3

Monoclonal antibodies specific for holo-TC II are immobilized on magnetizable particles. To an aliquot of serum (100-500 μ L) mixed with an equal volume of PBS is added a fixed amount of I^{125} labelled holo-TC II and a limited amount of the immobilized anti-holo-TC II

monoclonal antibody. After incubation for 15 min the magnetizable particles are sedimented by using a strong magnet. The particles are washed with PBS, and the radioactivity measured. The concentration of holo-TC II is determined from a standard curve.

EXAMPLE 4

Recombinant holo-TC II is immobilized on magnetizable particles. To an aliquot of serum (100-500 μ L) mixed with an equal volume of PBS is added a fixed amount of immobilized holo-TC II and an excess of monoclonal antibody specific for holo-TC II and radiolabelled with I^{125} . After 15 min incubation the particles are sedimented by using a strong magnet. The particles are washed with PBS, and the radioactivity measured. The concentration of holo-TC II is determined from a standard curve.

EXAMPLE 5

To an aliquot of serum (100-500 μ L) is added an excess of biotinylated cobalamin, so as to bind all apo-TC II and apo-HC. After incubation for 10 min, the serum sample is treated with avidin coated magnetic particles for 10 min. The particles are sedimented by using a strong magnet. The supernatant is recovered and mixed with two non-overlapping monoclonal anti-TC II antibodies, one radiolabelled with I^{125} and the other with conjugated biotin. After 10 min avidin-coated magnetizable particles are added and allowed to bind the biotinylated adducts for 10 min. Subsequently, the particles are sedimented with a strong magnet and washed with PBS. The radioactivity is measured and the holo-TC II concentration is determined by interpolation on a standard curve.

EXAMPLE 6

Same as Example 5, but the apo-TC II depleted serum

is mixed with a fixed amount I^{125} labelled holo-TC II and a limited amount of anti-TC II antibody immobilized on magnetizable particles. After incubation for 15 min the particles are sedimented by using a strong magnet.

The particles are washed with PBS and the radioactivity is measured.

Claims

1. An assay method for the determination of transcobalamin II bound cobalamin in a body sample, comprising contacting a cell free sample of a body fluid with an immobilised or immobilizable specific binding ligand for TC II or holo TC II, separating a ligand bound fraction from a non-ligand bound fraction and measuring the holo-TC II or TC-II bound cobalamin content therein.
2. An assay method as claimed in claim 1 wherein said specific binding ligands for TC II or holo-TCII allow for separation and concentration of the TC II or holo-TC II in the sample of at least 3-fold and up to greater than 10-fold.
3. An assay method as claimed in claim 1 wherein said assay is capable of detecting holo-TC II at a concentration as low as 9 pM.
4. An assay method as claimed in claim 1 wherein said assay is amenable to analysis by an automated process.
5. An assay method as claimed in claim 1 wherein said specific binding ligand is selected from the group comprising a polyclonal or monoclonal antibody, an antibody fragment, a polypeptide, an oligopeptide, a small organic chemical, a specific binder selected from a combinatorial chemistry or phage display library or a specifically binding sequence of DNA or RNA.
6. An assay method as claimed in claim 5 wherein said specific binding ligand is a monoclonal antibody.
7. An assay method as claimed in claim 1 wherein said specific binding ligand exhibits a high degree of

selectivity and specificity towards TC II and exhibits low affinity towards other TC proteins, in either apo or holo form, or any other cobalamin-binding protein.

8. An assay method as claimed in claim 1 wherein said method comprises contacting an immobilised or an immobilisable TC II or holo-TC II binding ligand with the sample under investigation;

separating a ligand-bound fraction from a non-ligand-bound fraction;

dissociating bound cobalamin from the holo-TC II molecules in the bound fraction and determining the concentration of cobalamin released, said dissociation being so affected that the concentration of the released cobalamin is at least 3 times and up to more than 10 times greater than the concentrations of holo-TC II in the initial sample.

9. An assay method as claimed in claim 8 wherein said cobalamin is released by changing the temperature or the pH of the surrounding medium.

10. An assay method as claimed in claim 1 wherein the different cobalamin forms are converted to the less light sensitive cyanocobalamin by treatment with KCN prior to contacting said sample with a specific binding ligand.

11. An assay method as claimed in claim 1 wherein said free cobalamin is determined by a competition assay performed by contacting an immobilised binding partner for cobalamin with the dissociated cobalamin of the sample in the presence of labelled ligand which competes with the isolated cobalamin for binding to the immobilised binding partners.

12. An assay method as claimed in claim 1 wherein the binding ligands for TC II are immobilised and bind to both holo- and apo-TC II.

13. An assay method as claimed in claim 1 wherein said method comprises contacting a solid support having immobilised thereon a TC II or holo-TC II binding ligand, with the sample under investigation and also with a non-immobilised ligand,

wherein said immobilised ligand is capable of binding to TC II or holo-TC II, to said non-immobilised ligand or to complexes of said TC II or holo-TC II and said non-immobilised ligand, and said non-immobilised ligand is capable of binding to at least one of said immobilised ligand, TC II or holo-TC II and complexes of said immobilised ligand and TC II or holo-TC II;

wherein if said assay method is a sandwich assay, at least one of said ligands is specific for holo-TC II and if said assay is a competition assay said immobilised ligand is specific for holo-TC II and competitors thereof;

whereby the proportion of said immobilised ligand bound by TC II or holo-TC II, by said non-immobilised ligand or by complexes of said non-immobilised ligand and TC II or holo-TC II is dependent on the amount of holo-TC II present in said sample, and,

said non-immobilised ligand is capable of generating a directly or indirectly detectable signal when bound or when unbound;

separating a bound fraction from a non-bound fraction; and

directly or indirectly determining the non-immobilised ligand bound to the immobilised ligand (the bound fraction) or non-bound and in solution (the non-bound fraction);

where the contacting of the sample and said non-immobilised ligand with the solid support may be

performed separately, simultaneously or sequentially, and if performed separately or sequentially, they may be contacted in either order.

14. An assay method as claimed in claim 13 wherein said non-immobilised ligand competes for binding to the immobilised ligand with the holo-TC II, a high level of unbound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of unbound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample.

15. An assay method as claimed in claim 13 wherein said non-immobilised ligand binds to the TC II or holo-TC II which is bound in turn to the immobilised ligand, such that a high level of bound non-immobilized ligand is indicative of a high concentration of holo-TC II in the sample and a low level of bound non-immobilized ligand is indicative of a low concentration of holo-TC II in the sample.

16. An assay method as claimed in claim 1 wherein a preliminary separation step is carried out using cobalamin or an analogue or fragment thereof which selectively binds the apo-forms of both TC II and haptocorrin (HC), such that the apo forms of the TC II and HC proteins are bound by the cobalamin, analogue or fragment thereof and separated from the holo-TC II and holo-HC complexes.

17. An assay method as claimed in claim 16 wherein said preliminary step is performed and thus in the subsequent analysis of the holo-TC II and holo-HC complexes, the immobilised or non-immobilised ligands may be specific for holo-TC II or TC II or competitors thereof.

18. An assay method as claimed in claim 16 wherein said cobalamin analogue or fragment thereof is tethered to a support by biotin.

19. An assay method as claimed in claim 16 wherein biotinylated cobalamin is added to the sample under analysis in a non-immobilised form where it binds to the apo-forms of TC II and HC, said sample then being contacted with a solid surface having a binding partner for the biotin immobilised thereon, such that a complex of binding partner-biotinylated cobalamin-TC II/HC then forms which can be easily isolated from the sample.

20. An assay method as claimed in claim 19 wherein said biotin binding partner is avidin.

21. An assay method as claimed in claim 16 wherein the holo-TC II pool is determined after separation by contacting the sample with an immobilised TC II ligand which captures the holo-TC II complex leaving the haptocorrin in solution and then contacting the immobilised holo-TC II with a second TC II ligand which is labelled and thus detectable.

22. An assay method as claimed in claim 16 wherein after separation, said holo-TC II molecules compete with labelled non-immobilised ligand for binding to immobilised ligand therefor and the amount of holo-TC II is determined in relation to the amount of labelled non-immobilised ligand bound or not bound to said immobilised ligand.

23. An assay method as claimed in claim 18 wherein the binding of apo TC II and apo HC to cobalamin, analogues or fragments thereof takes place at a site or in such a manner which inhibits subsequent recognition and binding of the immobilised cobalamin bound TC II by the non-

immobilised ligand or binding partner for TC II.

24. An assay method as claimed in claim 1 wherein a fraction is separated from the sample which comprises at least a portion of the desired TC II subset of proteins.

25. An assay method as claimed in claim 1 wherein said non-bound fraction is at least 80%, 90% or 95% free of either TC II or holo-TC II.

26. An assay method as claimed in claim 1 further comprising a preliminary separation step in which the sample is contacted with an immobilized or immobilizable specific binding ligand for haptocorrin.

27. An assay method as claimed in claim 1 wherein said TC II binding ligand possesses an affinity constant of at least 10^9M^{-1} .

28. An assay method as claimed in claim 27 wherein the affinity constant is greater than $2 \times 10^9 \text{M}^{-1}$.

29. An assay method as claimed in claim 28 wherein the affinity constant is greater than 10^{10}M^{-1} .

30. An assay method as claimed in claim 29 wherein the affinity constant is greater than 10^{11}M^{-1} .

31. An assay method as claimed in claim 1 wherein the degree of cross reactivity of a holo-TC II or TC II binding ligand with HC is less than 1%.

32. An assay method as claimed in claim 31 wherein the degree of cross-reactivity is between 0.1% and 1%.

33. An assay method as claimed in claim 32 wherein the degree of cross-reactivity is less than 0.1%.

34. An assay method as claimed in claim 1 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 3-fold.

35. An assay method as claimed in claim 34 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 5-fold.

36. An assay method as claimed in claim 35 wherein the binding ligands for TC II or holo-TC II concentrate the ligand by at least 10-fold.

37. An assay method as claimed in claim 1 wherein said sample comprising the holo-TC II complex is contacted with a solid phase to which a labelled ligand recognising the same binding sites on the immobilised ligands as holo-TC II is bound; holo-TC II in said sample competes with said bound labelled ligand for said binding sites such that after equilibration of the system, there is a directly proportional relationship between the amount of labelled ligand displaced from said solid support and detectable in solution and the amount of holo-TC II present in the original sample; said labelled ligand being detected directly or indirectly as the amount of labelled ligand bound or not bound to said solid support as appropriate.

38. An assay method as claimed in claim 1 wherein said holo-TC II containing sample is contacted with a solid support having holo-TC II immobilised thereon and a labelled non-immobilised holo-TC II specific binder, wherein free holo-TC II in the sample and immobilised holo-TC II compete for binding with the labelled non-immobilised ligand; and determination of the labelled ligand bound to the solid phase or remaining in solution allows determination of the holo-TC II concentration.

39. An assay method as claimed in claim 1 wherein said holo-TC II containing sample is contacted with labelled holo-TC II and immobilised ligand therefor; said labelled and non-labelled holo-TC II complexes compete for binding to the immobilised ligand and after equilibrium is reached, the amount of labelled holo-TC II bound to the immobilised ligand is indirectly proportional to the amount of holo-TC II in the sample.

40. An assay method as claimed in claim 1 wherein in a competition assay, the labelled holo-TC II may be detected directly or indirectly and may be determined as the amount of labelled holo-TC II bound or not bound to the solid support as appropriate.

41. An assay method as claimed in claim 1 wherein in an immunoassay, the labelled holo-TC II may be detected directly or indirectly and may be determined as the amount of labelled holo-TC II bound or not bound to the solid support as appropriate.

42. An assay method as claimed in claim 1 wherein said body sample is selected from the group comprising seminal fluid, cerebro-spinal fluid, amniotic fluid or a blood derived sample.

43. An assay method as claimed in claim 42 wherein said sample is serum or plasma.

44. An assay method as claimed in claim 1 wherein said bound fraction is separated from said unbound fraction by precipitation, centrifugation, filtration or chromatographic methods.

45. An assay method as claimed in claim 1 wherein said detectable ligand is labelled with a signal forming label which may be determined by luminescence,

chemiluminescence, colorimetric assessment, fluorescence, radioactivity or by enzymic activity.

46. An assay method as claimed in claim 1 wherein said immobilised ligand for one or the other components of the apo and/or holo TC II/HC complexes is arranged in a column to which the body fluid containing the TC II cobalamin complex is applied and contacted with the binding ligand(s).

47. An assay method as claimed in claim 1 wherein said the binding ligands used to separate a fraction of a sample are immobilised on a particulate solid phase support.

48. An assay method as claimed in claim 47 wherein said solid support comprises magnetic beads.

49. A kit for use in a diagnostic assay according to claim 1, comprising:

an immobilized or immobilizable specific binding ligand for TC II or holo-TC II;

preferably a holo-TC II solution of known concentration or a set of such solutions having a range of holo-TC II complex concentrations;

optionally, a release agent to release cobalamin from holo-TC II; and

optionally a labelled ligand.

ABSTRACT

The present invention relates to assay methods for the determination of cobalamin or vitamin B₁₂ in a body fluid and in particular to assay methods for the metabolically active pool of cobalamin, comprising contacting a cell free sample of a body fluid with an immobilised or immobilizable specific binding ligand for TC II or holo TC II, separating a ligand bound fraction from a non-ligand bound fraction and measuring the holo-TC II or TC-II bound cobalamin content therein.

DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention (Design, if applicable) entitled: **ASSAY**, the specification of which (check one):

☐ is attached hereto, or ☒ was filed on: **December 1, 1998** as U.S. Application Number or PCT International Application

Number:

and (if applicable) was amended on:

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56*. I hereby claim foreign priority benefits under *Title 35, United States Code §119* of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)			PRIORITY CLAIMED	
Number	Country	Day/Month/Year Filed	Yes	No
9820473.8	UK	18 September 1998	X	

☐ Additional Priority Application(s) Listed on Following Page(s)

I HEREBY CLAIM THE BENEFIT UNDER TITLE 35 U.S. CODE §119(E) OF ANY U.S. PROVISIONAL APPLICATIONS LISTED BELOW.	
Application Number	Day/Month/Year Filed

☐ Additional Provisional Application(s) Listed on Following Page(s)

I hereby claim the benefit under *Title 35, United States Code, §120* of any United States application(s) or PCT international application(s) designating The United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of *Title 35, United States Code, §112*, I acknowledge the duty to disclose information which is material to patentability as defined in *Title 37, Code of Federal Regulations, §1.56* which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

Application Number	Filing Date	Status - Patented, Pending or Abandoned

☐ Additional US/PCT Priority Application(s) listed on Following Page(s)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under *section 1001 of title 18 of the United States Code* and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I (We) hereby appoint as my (our) attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: J. Ernest Kenney, Reg. No. 19,179; Eugene Mar, Reg. No. 25,893; Richard E. Fichter, Reg. No. 26,382; Charles R. Wolfe, Jr., Reg. No. 28,680; Thomas J. Moore, Reg. No. 28,974; Joseph DeBenedictis, Reg. No. 28,502; Benjamin E. Urcia, Reg. No. 33,805; and

I(we) authorize my(our) attorneys to accept and follow instructions from FRANK B. DEHN & CO. regarding any matter related to the preparation, examination, grant and maintenance of this application, any continuation, continuation-in-part or divisional based thereon, and any patent resulting therefrom, until I(we) or my(our) assigns withdraw this authorization in writing.

Send correspondence to: **BACON & THOMAS**
625 Slaters Lane - 4th Floor
Alexandria, VA 22314-1176

Telephone Calls to: (703) 683-0500

FULL NAME OF FIRST OR SOLE INVENTOR Erling SUNDREHAGEN	CITIZENSHIP Norwegian
RESIDENCE ADDRESS <i>28-282</i> Ulven 87c, 0510 Oslo, NORWAY	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE Jan 3, 1999	SIGNATURE <i>E. Sundrehagen</i>

☒ See following page(s) for additional joint inventors.

CONTINUATION OF DECLARATION FOR PATENT APPLICATION AND APPOINTMENT OF ATTORNEY

Page 2

FULL NAME OF JOINT INVENTOR Lars ÖRNING	CITIZENSHIP Swedish
RESIDENCE ADDRESS Bogstadveien 24 N-0355 OSLO, NORWAY	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE 28.12.98	SIGNATURE <i>Lars Ö</i>

FULL NAME OF JOINT INVENTOR	CITIZENSHIP
RESIDENCE ADDRESS	POST OFFICE ADDRESS IS THE SAME AS RESIDENCE ADDRESS UNLESS OTHERWISE SHOWN BELOW
DATE	SIGNATURE

FULL NAME OF JOINT INVENTOR	CITIZENSHIP
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☐ See following pages for additional joint inventors/priority applications.